European stone fruit Yellows phytoplasma in Japanese plum and Myrobalan plum in Bosnia and Herzegovina

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Abstract

Stone fruits from commercial as well as abandoned orchards were evaluated for European Stone Fruit Yellows phytoplasma (ESFY) presence during 2004-2007 years. Orchards were monitored in western and southern districts of Bosnia and Herzegovina. In the first survey conducted in period of 2004 till 2005 the causal agent of ESFY was identified on peach *(Prunus persica*) and apricot (*Prunus armeniaca*) plants in both surveyed districts. During 2007, a new survey was performed and samples were taken from symptomatic and symptomless plants of European plum (*Prunus domestica*), Japanese plum (*Prunus salicina*), Myrobalan plum (*Prunus cerasifera*) and cherry (*Prunus avium*). Samples were analyzed using real-time PCR and nested PCR approaches. In this extended survey, the presence of ESFY phytoplasma was additionally identified in Japanese plum and myrobalan plum trees.

Keywords: Bosnia and Herzegovina, myrobalan plum, Japanese plum, phytoplasma, ESFY, PCR

Introduction

Stone fruits are traditionally grown in Bosnia and Herzegovina (B&H), among which the main species is European plum. Two types of fruit orchards nowadays exist: old inherited fruit orchards from the previous state farms (many completely abandoned and often with a few scattered trees) which are still the dominant type of orchards and plantings more recently established. For further development of the fruit tree industry and the establishment of a certification program in the last ten years, the sanitary status of fruit trees started to be assessed in detail. The first survey for the presence and distribution of European stone fruit yellows (ESFY; 'Candidatus *Phytoplasma prunorum*') was conducted in period 2004 till 2005. First results on the identification of the ESFY causal agent in peach and apricot trees as well as on vector *Cacopsylla pruni* was reported by Delic et al. (2007). This paper provides a continuation of the ESFY study in B&H.

Materials and methods

Field survey: The survey was organized in the North-Western of the country, an area important for stone fruit production. Field visits and sampling occurred in second part of August 2007. Leaf samples were collected from symptomatic and symptomless European plum (*Prunus domestica*), Japanese plum (*Prunus salicina*), myrobalan plum (*Prunus cerasifera*) and cherry (*Prunus avium*) trees. Our attention was focused on one orchard in the Banjaluka region with Japanese plum and myrobalan plum species showing phytoplasma symptoms. Mid-ribs extracted from five samples (2 Japanese plum, 1 myrobalan plum, 1 European plum and 1 cherry) were analyzed.

Total DNA extraction: Automated DNA extraction procedure was performed (Boben et al., 2007; Pirc et al., 2009). DNA was isolated from 200 mg of homogenized material from fruit tree samples using the QuickPick™ Plant DNA kit (BioNobile, Finland) and KingFisher mL (Thermo Scientific, USA) machine. Final elution was performed in 200 µl of sterile double distilled water.

Molecular analyses: The presence of phytolasma in DNA samples was checked using two molecular approaches: nested PCR followed by RFLP analyses and real-time PCR. PCR was performed using the universal phytoplasma P1/P7 primers (Schneider et al., 1995) slightly modified according to Hren et al. (2007), followed by nested PCR using AP group specific primers f01/r01(Lorenz et al., 1995). Products were visualized on 1% agarose gel, stained with ethidium bromide. All positive fo1/ro1 PCR products were then analysed by RFLP using the restriction enzymes *Ssp*I (Promega, USA) and *Bsa*AI (New England, BioLabs). Real-time PCR assay was done according to the procedure described by Hren et al. (2007) using UniRNA phytoplasma non-specific primers and a TaqMan probe. In the same run, the DNA extraction procedure was checked by using the 18S rRNA TaqMan assay (Applied Biosystem, USA). All real-time PCR reactions were run in 10µl reaction volumes under standard conditions in a 7900 HT Sequence Detection System

(Applied Biosystem, USA). The results of amplification were analyzed using SDS 2.2 software (Applied Biosystem, USA).

Results

Typical symptoms of ESFY were observed on the phytoplasma positive Japanese plum and myrobalan plum. In Japanese plum, symptomatic leaves were rolled longitudinally upwards, and red colored. Red colored leaves as well as red colored leaf veins were found on myrobalan trees. Real–time PCR analyses detected phytoplasmas in two of the five samples tested, one Japanese plum and one myrobalan plum (Table 1). Average Ct values obtained amplifying 18S rRNA confirmed efficacy of the extraction procedure. AP group specific nested PCR products were obtained in the same real-time PCR positive samples. RFLP analyses determined the presence of 'Candidatus *Phytoplasma prunorum*'.

Tab. 1 Nested PCR and real-time PCR results of tested samples

Sample type	Symptoms observed[a]	Nested PCR results	RFLP	Real-time PCR[b] (UniRNA)	Real-time PCR[b] (18S rRNA)
Japanese plum	sl; rl; yl; rv	negative	/	undetermined	19,5
Japanese plum	ur; rl; rv; cl	positive	'Ca. *P. prunorum'*	22,5	15,6
Myrobalan plum	rl; rv;cl	positive	'Ca. *P. prunorum'*	25,9	17,8
European plum	rv; bl; cl	negative	/	undetermined	18,2
Cherry	rv; cl	negative	/	undetermined	16,6

[a]sl-small leaves; ur- longitudinally upward rolled leaves; rl-red colored leaves; rv-red colored leaf veins; yl-yellow colored leaves; cl-crıspy leaves; bl-bronze colored leaves; rv-reduced tree vigor.
[b] Ct are expressed as the mean value from a triplicate

Discussion

Infected Japanese plum and myrobalan plum were young trees of three-four years old. Symptoms observed on Japanese plum were in line with the literature describing ESFY symptoms on this host (Kison and Seemüller, 2001). On the other hand, unusually prominent phytoplasma symptoms were observed on myrobalan plum. Myrobalan plum is usually symptomless or infected trees can showed slight leaf chloroses. However, symptom expression in trees infected with phytoplasma depends on many factors and the most important are host cultivar/rootstock combination and genetic variability among pathogens strains (Kison and Seemüller, 2001). Recently, Martini et al. (2009) characterized 'Candidatus *Phytoplasma prunorum*' strains using *aceF* gene. Results obtained using two restriction enzymes in a PCR/RFLP method differentiated 'Ca. *P. prunorum*' strains into five different RFLP subgroups. Although the symptoms on infected myrobalan plum could be a consequence of other biotic or abiotic factors, it would be interesting to further characterize the present isolates. This limited survey demonstrated the presence of ESFY phytoplasma in Japanese plum and myrobalan plum in Bosnia and Herzegovina. Nevertheless, to have a clear insight into the distribution as well as the strain composition in the country, extensive surveys will need to be done. The distribution of ESFY in different *Prunus* species and varieties still have to be investigated in B&H. As the presence of the vector *Cacopsylla pruni* is confirmed, (Delic et al., 2007), ESFY could be another major disease and threat for the local stone fruit industry in the future.

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